

# Ethylene Biosynthesis from 1-Aminocyclopropanecarboxylic Acid

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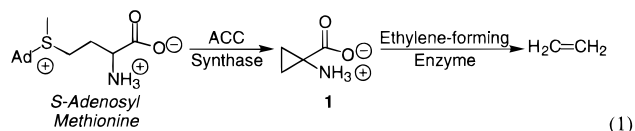
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## Introduction

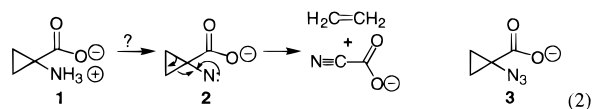
It is very surprising for a chemist to consider that the simplest alkene has biological activity, but indeed ethylene is a very significant plant hormone.<sup>1</sup> When this property is known at all outside the plant physiology community, it is usually associated with fruit ripening. Ethylene mediates several other biological processes, including germination of seeds, senescence (wilting and other catabolism, such as the Fall colors), abscission (dropping of Fall leaves), and responses to environmental stress. The “old wives’ tale” about hastening ripening by placing unripe fruit into a paper bag with ripe fruit is quite true, and works simply because it limits the diffusion of naturally produced ethylene gas. So-called climacteric fruit (apples, tomatoes, and melons, inter alia) undergo a profound change in physiological activity that initiates ripening, and use ethylene, acting at its receptor, to trigger their tissues. It has long been known that this ethylene can also affect other fruit. A Latin proverb<sup>2</sup> states “uvaeque conspecta livorem ducit ab uva”—“a grape assumes a sickly hue from a grape it has seen”. This points up the downside of ethylene action, that over-ripening leads to tremendous losses of agricultural products. Materials that moderate the production of ethylene could thus have profound value in plant physiology and agronomy. To place the search for such substances on a rational basis, ethylene biosynthesis must be understood. Before 1979, it was known that methionine provides the carbons of ethylene, but exactly how this amino acid was converted to the hormone was unknown. Chemical investigations of ethylene biosynthesis were spurred by Yang’s<sup>3</sup> discovery that the immediate biosynthetic precursor to ethylene is

1-aminocyclopropanecarboxylic acid (ACC, **1**), which is derived from *S*-adenosylmethionine through the action of the enzyme ACC synthase. This Account summarizes research in our laboratories at Stanford and Duke addressing the process by which ACC is converted to ethylene.



## Model Chemical Reactions

There were several early speculations for processes that might convert ACC to ethylene, none limited by data<sup>4</sup> or reasoning based on accepted chemical mechanisms.<sup>2,5</sup> Because bioorganic chemistry has long benefited from model systems that mimic biological transformations, two were considered in our laboratory. One was based on the idea that a cyclopropyl nitrene **2** might be generated from the amino acid.<sup>6</sup> The chemistry of cyclopropyl nitrenes was

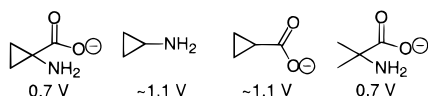


known. It leads, in a concerted, 6-electron chelotropic process that adheres to conservation of orbital symmetry, to the formation of an alkene plus a nitrile. In this case, this would result in ethylene plus cyanoformic acid. We prepared a reasonable precursor of the nitrene, azidoacid **3**, and decomposed it under photochemical, thermal, and acidic conditions. The latter presumably generates the nitrenium ion. In all cases, ethylene is produced. Since cyanoformic acid is unstable with respect to its constituents, cyanide and CO<sub>2</sub>, we detected the other products of the decomposition of **3** by ion-selective electrode and gas chromatography, respectively, in stoichiometric amounts. At this time, the fate of the other carbons of ACC in the biosynthetic process had not been determined, though it was known that the carboxyl group of methionine is released as CO<sub>2</sub>; presumably the same would be true of ACC.

The second model reaction we considered was a single-electron-transfer process, which was suggested by other research then ongoing concerning the reaction of cyclopropylamines with oxidase enzymes such as monoamine oxidase and cytochrome P450.<sup>7</sup> Such single-electron-transfer reactions can be readily mimicked by electrochemical oxidation. Investigation of the electrochemistry of ACC and two related compounds,<sup>8</sup> cyclopropanecarboxylic acid and cyclopropylamine, by cyclic voltammetry showed a surprising difference: ACC is easier to oxidize than either by 400 mV<sup>9</sup> (Chart 1). This difference in oxidation potential was shown to be related not to the

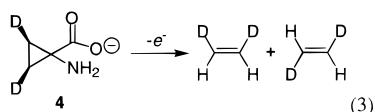
Michael C. Pirrung received a B.A. in chemistry from the University of Texas—Austin in 1975 and a Ph.D in organic chemistry (with Clayton Heathcock) at the University of California—Berkeley in 1980. After postdoctoral study (with Gilbert Stork) at Columbia, he began his academic career at Stanford. In 1988, he joined a working group of scientists meeting in Palo Alto to form a then-new company, Affymax, which was an early contributor to developments in combinatorial chemistry. In 1990, he moved to Duke University where he is now Professor of Chemistry. A sabbatical at the Baylor College of Medicine in 1995 resulted in one current research interest, DNA chip fabrication and use in genetic analysis.

Chart 1



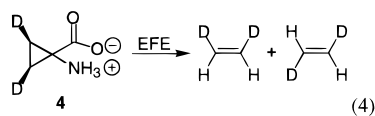
cyclopropane but to the dual amino and carboxylate functions, as  $\alpha$ -aminoisobutyrate (AIB) shows an oxidation potential similar to that of ACC. While the single-electron-transfer mechanism for reaction of cyclopropylamines with the iron-oxo intermediate in cytochrome P450 or the flavin in monoamine oxidase has been criticized because the thermodynamics do not seem favorable, ACC would react much more easily with such oxidants.

Two other experiments were performed with this electrochemical model. One was a bulk electrolysis and product study. When oxidized to completion, ACC shows the 2/3 F/mol coulometry usually observed in 2-electron primary amine oxidations. Ethylene is produced in this reaction in a modest yield; interestingly, cyanide is produced in the same amount, stimulating the investigation of cyanide production described later. When cyclopropylamine is subjected to similar electrochemical oxidation, ethylene is produced in comparable yield, suggesting that the amine is the site of ACC oxidation. The second part of the electrochemistry study focused on possible mechanisms. On oxidation of the synthetic, stereospecifically dideuterated ACC **4**, a 1:1 mixture of *cis*- and *trans*-ethylene- $d_2$  is produced (FT IR). This result shows that the electrochemical process involves stepwise cleavage of the two cyclopropane bonds, as contrasted with the nitrene reaction, which we believe is concerted (vide infra).



## Stereochemistry

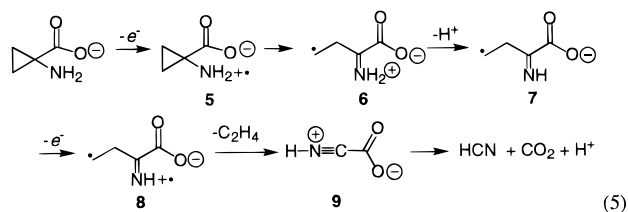
With the deuterated amino acid **4** in hand, the stereochemistry of the biosynthetic process was likewise examined. As there was not even a cell-free system that produced ethylene at this time, this experiment was performed in plant tissue. In apple, cantaloupe, and mung bean, compound **4** is converted to a 1:1 mixture of the deuterated ethylenes. At about the same time, Baldwin's



laboratory at Oxford reported similar experiments in apple using both stereoisomers of the dideuterated ACCs.<sup>10</sup> Baldwin also studied another model system for ACC oxidation, using NaOCl, which proceeds with retention of configuration. This presumably occurs via N-chlorina-

tion and  $\alpha$ -elimination to form the cyclopropyl nitrene, which undergoes concerted fragmentation as described above.

Having in hand both model chemistry and the biosynthetic result with **4**, as well as substantial precedents from the monoamine oxidase and cytochrome P450 literature, it was simple for us to propose a mechanism for the electrochemical model that must be closely related to ethylene biosynthesis. Key is the production of the cyclopropylamine radical cation **5**. On the basis of known



radical clock chemistry,<sup>11</sup> cyclopropylamine radical cations were expected to undergo rapid ring opening, and subsequent research (described later) has confirmed this. Free rotation in the resulting intermediate **6** accounts for the loss of stereochemistry. From **6** forward, the mechanism of ethylene production is more difficult to surmise; indeed, the steps that were proposed in 1982 are ones that, from a purely chemical perspective, most would not advocate, though they fit the stoichiometry. Another electron must be lost from **6**, along with a proton. The modest efficiency of this biomimetic ethylene production might reflect a mechanistically nonoptimal pathway involving highly reactive intermediates unconstrained by an enzyme.

## Cyanide

At the outset of our work, it was believed that the products of ethylene biosynthesis from C2 and N of methionine were formic acid and ammonia. The knowledge that ACC is an intermediate in the conversion of methionine to ethylene might not alter this view, but the fact that both of the model processes we had examined produced cyanide was strong impetus to reinvestigate the products from this sector of ACC. The  $^{13}\text{C}$ -labeled derivative was prepared and incubated in apple tissue.<sup>12</sup> Because this was an *in vivo* experiment, existing metabolic detoxification pathways for cyanide, which were well-known, came into play. Conn showed that cyanide is incorporated in plants into the nonproteinogenic amino acid  $\beta$ -cyanoalanine (**10**),<sup>13</sup> which can be further converted into asparagine. Incorporation of  $^{13}\text{C}$  from ACC into C-4 of either  $\beta$ -cyanoalanine or asparagine was assessed via a cocktail consisting of asparaginase and glutamate-oxaloacetate transaminase in the presence of zinc, which liberates C-4 as  $\text{CO}_2$  for analysis (6). The  $^{13}\text{C}$  enrichment in this  $\text{CO}_2$  (GC-MS) confirmed production of cyanide stoichiometric with ethylene. At about the same time, a collaboration

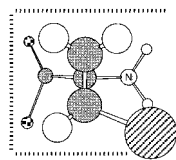
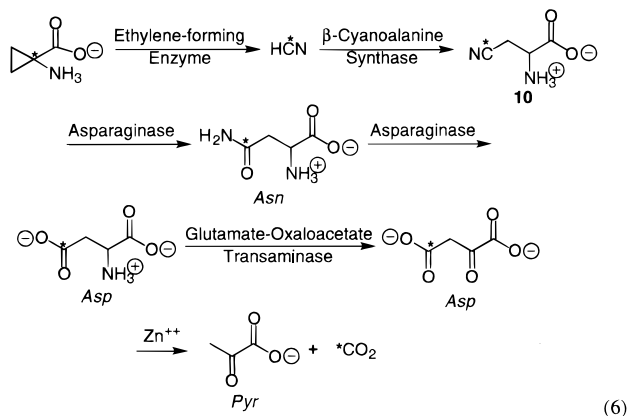


FIGURE 1. Postulated shape of the EFE active site.

between Walsh and Yang reported similar findings in mung bean and vetch.<sup>14</sup>

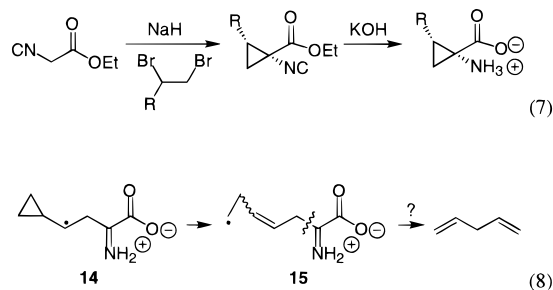


## Substrate Analogues/Inhibitors

Structure–activity relationships contribute greatly to the analysis of macromolecule–small molecule interactions, and substrate analogues/inhibitors related to ACC have provided useful insight into ethylene biosynthesis. Early on, Yang and Ichihara<sup>15</sup> found that *allo*-coronamic acid (**12**, 2-ethyl-ACC) (Chart 2) is preferred over its other stereoisomers as a substrate for the production of 1-butene in plants. This diastereoselectivity was significant as the first demonstration that an enzymatic process must be involved in ethylene biosynthesis, rather than some adventitious chemical/free-radical oxidation process, to which ACC is in fact quite sensitive.<sup>16</sup> The rate of butene production from **12** is <30% relative to ethylene production from ACC. Our laboratory extended this work with the preparation of **11**, 2-methyl-ACC, from compounds of known absolute configuration,<sup>17</sup> and demonstrated that substrates with the (1*R*,2*S*) absolute configuration shown are preferred. Relative to ethylene production from ACC, the rate of propylene production from **11** is 80%, which is a  $k_{\text{cat}}$  effect. Thus, only one position on the ring may be substituted, and increased steric demand of this substituent decreases activity. These data enabled us to propose a crude shape for the enzyme active site (Figure 1). Around this time, Baldwin reported similar findings (including deuterium labeling that enabled stereochemistry to be more deeply analyzed).<sup>18</sup>

The method we developed to synthesize specifically the active alkyl-ACC diastereomer uses the cycloalkylation of

ethyl isocyanoacetate with 1,2-dibromides developed by Schöllkopf.<sup>19</sup> It was used to prepare cyclopropyl-ACC (**13**),<sup>20</sup> which was designed as a probe for intermediate **6** in our postulated mechanism. It was expected on the basis



of earlier radical clock research that the cyclopropyl-substituted radical **14** would rapidly undergo a further ring opening. When **13** was exposed to plant tissue, the only C5 product detected by MS was 1,4-pentadiene. The efficiency of this process is low compared to that of ethylene production from ACC, though the cyclopropyl group does not greatly inhibit the ability of **13** to access the active site, since its  $K_i/K_m = 3$ . We attribute this difference to an odd free-radical step that must be included in the postulated mechanism to reach the observed product. If a homolytic cleavage analogous to (5) is applied to **15**, it is necessary to invoke a rare 1,2-hydrogen shift to yield 1,4-pentadiene. A consequence of this unfavorable reaction pathway may be the fact that **13** is a mechanism-based or “suicide” inactivator of ethylene production. A radical such as **15**, with no favorable unimolecular reaction pathway available, may react with key enzymatic residues. This experiment also limits the lifetime of radical **6**, which could not give nonradical products (i.e., ethylene) at a rate  $>10^8 \text{ s}^{-1}$  without producing vinylcyclopropane from **13**.

Other analogues we have studied include hydroxymethyl-ACC (**16**),<sup>21</sup> which was prepared for use in affinity tagging and affinity purification procedures, and the cyclopropene analogue **17**<sup>22</sup> (Chart 3). The former is a good inhibitor, with a  $K_i/K_m$  of 1. As one might project, the latter is converted to acetylene by plant tissue, but at only 0.2% of the rate that ACC is converted to ethylene. It is one of the best competitive inhibitors, with  $K_i/K_m = 0.6$ , showing that it can access the active site and again suggesting that a radical intermediate in its processing may be diverted. Like **13**, it shows mechanism-based inactivation. While a simple extrapolation of the mechanism in (5) would lead to vinyl radical **20** that could be the inactivating species, it is also possible that conjugation promotes decarboxylation to **18** (9). This radical could itself inactivate, or be oxidized to a very electrophilic aromatic cyclopropene iminium ion.

Chart 2

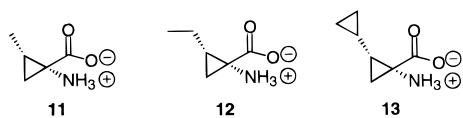
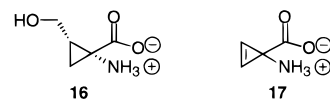
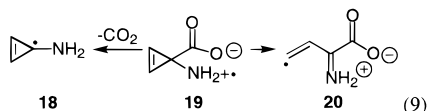


Chart 3





After we had purified the ethylene-forming enzyme (EFE; vide infra), it became much easier to gain hard data on analogues. A number of compounds were studied, one class being the amino acid hydroxamates (Chart 4), which were chosen because of the known dependence of enzymatic activity on iron. It had been supposed for some time that iron is at the active center of the EFE. The differential activities of **20–23** demonstrate that they do not merely serve as iron chelators to remove the required metal from the enzyme. The increase in affinity as alkyl groups are added to the  $\alpha$ -carbon of glycine and then the ring is closed suggests that the enzyme has a hydrophobic pocket optimized to bind the cyclopropane. When only a single methyl group is present, the energetic cost is 2.4 kcal/mol vs the cyclopropane, and when there is no alkyl substituent, the cost is 2.8 kcal/mol. These values can be related to work by Matthews<sup>23</sup> concerning the effect of internal voids in proteins on their folding thermochemistry. In those studies, the energetic cost of replacing, e.g., a valine with an alanine was determined. Matthews' data suggest that each angstrom<sup>3</sup> of free space results in an energy loss of  $\sim 24$  cal/mol. Using this value, we calculate that the volume of the cyclopropane binding site in the EFE is 117 Å<sup>3</sup>. The most potent inhibitor, ACC-hydroxamate (**23**), is also a substrate for ethylene production. Compared to ACC, its Michaelis constant is increased by  $\sim 3\times$  and its turnover is decreased by  $\sim 7\times$ . Comparison of its  $K_i$  when ACC is the substrate and the  $K_m$  when it is the substrate reflects the difference between the inhibitor dissociation constant and the Michaelis constant when the  $k_{cat}$  step is fast relative to dissociation.<sup>24</sup> That is,  $K_m$  is the same as  $K_s$ , the dissociation constant of the E·S complex (and, in this case,  $K_i$ , the dissociation constant of the enzyme–inhibitor complex), only when the rate of reversal of E·S is much faster than the  $k_{cat}$  step (Chart 5). Thus, **23** is a “sticky” substrate that is much less likely to dissociate once the E·S complex is formed than to go forward.

## Further Mechanistic Study

Our laboratory next focused on two issues, refinement of the sequential single-electron-transfer mechanism and estimating some rates in it. The first issue was addressed by semiempirical MO calculations.<sup>25</sup> From this study we learned that the HOMO of radical **7** is not the nitrogen lone pair but the SOMO of the carbon radical, making it unlikely that the second electron oxidation gives **8**. It is also not very likely (from a chemical perspective) to oxidize this radical to a primary cation, requiring a new reaction pathway to be postulated. The second issue was addressed by determination of the secondary isotope effect for ethylene-*d*<sub>4</sub> production from ACC-*d*<sub>4</sub>.<sup>26</sup> This was accomplished by competition of deuterated and nondeuterated substrates, with GC–MS analysis. An effect as large

Chart 4

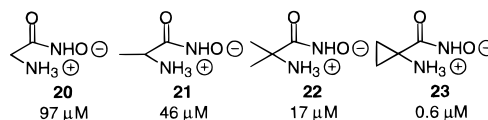
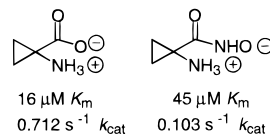
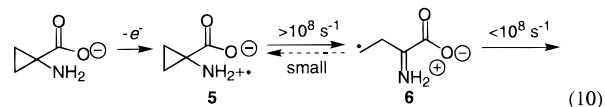


Chart 5

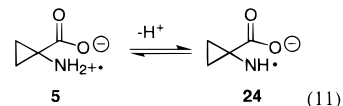


as 1.1 theoretically might be observed, but in fact no isotope effect was seen in either the biosynthetic or electrochemical model reactions. Because of the intricacies of isotope effects in Michaelis–Menten kinetics, competition experiments do not give information concerning the rate-determining step of the reaction, but rather on the “commitment factor” (effectively, the  $k_2/k_{-1}$  ratio for E·S) of the isotope-sensitive step (here, cyclopropane ring opening). Commitment is high, which makes intuitive chemical sense in that ring opening is exothermic (MNDO suggests  $\Delta H_{rxn}(\mathbf{5} \rightarrow \mathbf{6}) \cong 7$  kcal/mol) and favorable compared to back electron transfer. We were thus able to suggest the rates of processes in the early stages of ethylene production (10). The ring opening rate



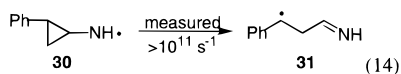
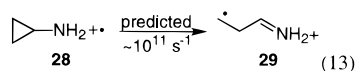
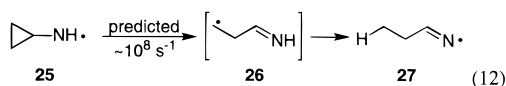
of a cyclopropylaminium ion was unavailable at this time, but  $>10^8$  s<sup>-1</sup> was a reasonable estimate based on known cyclopropylaminyl radical clocks (vide infra). Intermediate **6** cannot go forward faster than  $10^8$  s<sup>-1</sup>; if that were so, radical **14** could not undergo ring opening and vinylcyclopropane would be produced from **13**.

Recent physical organic studies from other laboratories have refined our thinking about this mechanism. One is a concern that the aminyl radical **24** is involved rather than radical cation **5** (11). The aminyl radical and radical



cation differ only by protonation, with the  $pK_a$  of dialkylaminyl radicals being about 7.<sup>27</sup> Because the pH environment of the active site is unknown, it would be difficult to exclude a priori the aminyl radical as an intermediate. On the other hand, EPR studies by Williams of cyclopropylamine radical cation and aminyl radical (**12**, **13**)<sup>28</sup> have shown that opening of **25** leads quickly to internal H-atom transfer from the N–H group to generate iminyl radical **27**. This species would not be competent in ethylene production, as it bears an ethyl unit, not an ethylene unit. Ring opening of radical cation **28** produces the stable **29**. The contrasting chemical behaviors of **25** and **28** therefore disfavor aminyl radical mechanisms.





On the basis of Newcomb's studies of amine radical cations,<sup>29</sup> it is possible to estimate the rates of these ring opening processes. 2-Phenylcyclopropylaminyl radical (**30**) opens to the stabilized radical **31** at  $>10^{11} \text{ s}^{-1}$ . Newcomb's earlier studies and known effects of phenyl groups on radical clock rates suggest that cyclopropylaminyl radical (**25**) opens  $\sim 10^3 \text{ s}^{-1}$  slower. Since amine radical cations can undergo rearrangements up to  $10^3$  faster than their cognate aminyl radicals, this suggests ring opening to produce **29** occurs at  $\sim 10^{11} \text{ s}^{-1}$ . The rate of the **5**  $\rightarrow$  **6** conversion in (10) may therefore be revised upward.

## Molecular Biology

In the late 1980s, ethylene research had come almost to a standstill, as most of the experiments that could be performed by feeding labeled/modified substrates to plant tissues had been accomplished. A leap forward into direct enzymatic studies was needed. Through reverse biochemistry experiments utilizing both mRNA function studies and "knockout" of ripening-related genes in transgenic tomatoes, Boller and Grierson cloned and sequenced a putative gene for the ethylene-forming enzyme.<sup>30</sup> At about the same time, a number of labs reported cell-free systems that produce ethylene by a valid pathway,<sup>31</sup> as shown by the discrimination between alkyl-ACC analogues that has become the hallmark of the enzymatic process.

We wished to extend these observations to the isolation of the homogeneous enzyme. Through a GenBank search on the putative EFE sequence determined by Boller, we identified a family of proteins with two regions of sequence homology (Figure 2). Some with known function (IPNS, DAOCS) are bacterial enzymes in the biosynthetic pathway to  $\beta$ -lactam antibiotics as well as members of the class of mononuclear non-heme iron enzymes.<sup>32</sup> Some members of the class use  $\alpha$ -ketoglutarate, which is oxidized to  $\text{CO}_2$  and succinate, as a cosubstrate. While the homology of the EFE to the bacterial enzymes was useful (and known in other laboratories), key to our purification of the ethylene-forming enzyme was the identification of a homologous sequence for hyoscyamine-6 $\beta$ -hydroxylase (H6H). This enzyme is involved in the biosyntheses of tropane alkaloids in plants, and its sequence had been obtained by purification to homogeneity of the protein from *Hyoscyamus* roots followed by Edman sequencing.<sup>33</sup> This work provided a clear precedent for purification of

F3H	178	<b>HTDPGTITLLQD</b>	190
H6H	217	<b>HYDGNLITLLQD</b>	229
EFE	177	<b>HSDAGGIILLFQD</b>	189
DAOCS	184	<b>HYDLSTLTLVHQT</b>	196
IPNS	214	<b>HEDVSLITVLYQS</b>	252
F3H	257	VVNLGDHGHFLSNGRFKNAD <b>HQA</b>	279
H6H	254	VVNLGLTLKVITNEKFEGSI <b>HRV</b>	276
EFE	214	VINLGDQIEVITNGKYKSV <b>HRV</b>	236
DAOCS	224	VVFCGAVGTLAIGGKVKAPK <b>HRV</b>	246
IPNS	254	LINCGSYMAHITDDYYPAPI <b>HRV</b>	276

**FIGURE 2.** Regions of sequence homology among flavanone-3-hydroxylase, hyoscyamine-6-hydroxylase, deacetoxycephalosporin C synthase, isopenicillin N synthase, and EFE. Conserved histidines, putative iron ligands, are shown in bold.

105...**LEEYRKT**MKEFAVELEK**LK**ALLDL**LL**CEN...134  
 ↑ ↑ ↑ ↑ ↑ ↑ ↑

**FIGURE 3.** Secondary structural analysis of the EFE reveals an amphipathic  $\alpha$ -helix, with hydrophobic residues indicated by arrows.

the ethylene-forming enzyme.<sup>34</sup> Simple steps, such as slicing plant tissue directly into liquid nitrogen, as had been done for H6H, and the inclusion of ascorbic acid in lysis buffer, as had been done for cell-free ethylene-forming systems, greatly increased recovery of active enzyme. Purification was achieved by conventional chromatographic methods, with use of a hydrophobic interaction column suggested by the identification, through secondary structural analysis of the protein sequence, of an amphipathic  $\alpha$ -helix (Figure 3). Three purification stages led to homogeneous ethylene-forming enzyme. Interestingly, the purification factor is only  $\sim 20$ – $30$ ; the EFE thus constitutes a significant fraction of the protein in apples. It is rather unstable, however, particularly in the presence of oxygen and its iron cofactor; during turnover, all activity is lost within 2 h. Electrospray mass spectrometry shows that the enzyme is posttranslationally modified, with the initiator methionine removed and the N-terminus acetylated. These data were consistent with research from other laboratories also isolating the EFE and showing it could not be sequenced by Edman degradation.<sup>35</sup> Yang found the enzyme is activated by  $\text{CO}_2$ , while we showed that its activity is enhanced by bicarbonate (up to 5 mM). This might involve carbamate formation, though efforts to establish this point have not been fruitful. Another possibility is that bicarbonate is involved in the assembly of the active redox center. Yang also reported that ascorbate is involved stoichiometrically in the conversion of ACC to ethylene. It is difficult to imagine ascorbate and bicarbonate in the inner coordination sphere of iron along with the substrate and protein ligands.

## The Question of Hydroxylation

Our laboratory has continued investigation of the radical-based mechanism for ethylene production in the face of persistent suggestions concerning the hydroxylation or

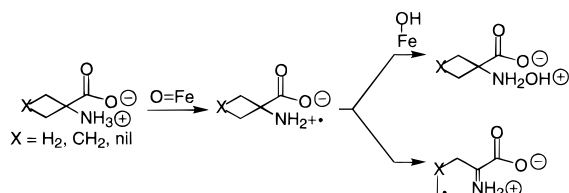
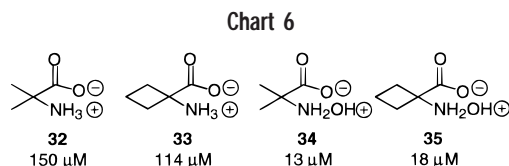
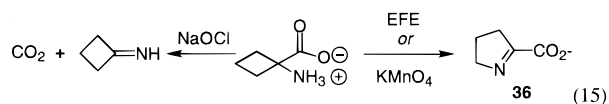


FIGURE 4.



nitrenium/nitrene pathway.<sup>36,37</sup> As the EFE is a presumptive iron–oxo enzyme, the crux of this matter is in our view the partitioning of a radical pair (composed of the amine radical cation of the substrate and an Fe–OH species) between ring opening (vide supra) and hydroxyl rebound (Figure 4). The hydroxyl rebound rate has not been determined for any mononuclear non-heme iron enzyme, but is likely slower than the  $10^{13} \text{ s}^{-1}$  observed in cytochrome P-450,<sup>38</sup> making it at least comparable to ring opening ( $\sim 10^{11} \text{ s}^{-1}$ ). While we were unable to prepare *N*-hydroxy-ACC to directly address this question, we recently examined enzymatic reactions of a substrate with slightly lower ring strain than ACC. Aminocyclobutanecarboxylic acid (ACBC, **33**) (Chart 6) should therefore favor *N*-hydroxylation because ring opening should be  $\sim 10^3 \text{ s}^{-1}$  slower than with ACC, whatever its rate.<sup>39</sup>

Neither of the *N*-hydroxyamino acid analogues (**34**, **35**) of ACBC or AIB (**32**) were detected in incubations of the amino acids with the EFE, but they were found to be much more potent inhibitors of ethylene production than the amino acids. This result could either support the hydroxylation pathway (because hydroxylamines bind tightly, but those which do not have the cyclopropane ring are not substrates) or refute it (because hydroxylamines are inhibitors, not substrates). The processing of labeled ACBC resolved this dilemma, as it is converted to  $\Delta^1$ -pyrroline-2-carboxylic acid (dehydroproline, **36**). The intrinsic chemical reactivity of ACBC under model reaction conditions was then examined. A chemical oxidant that uses a one-electron mechanism (based on loss of stereochemistry in ACC- $d_2$  oxidation) was chosen.<sup>16</sup> Permanganate converts ACBC to dehydroproline (**15**); decarboxylation to cyclobu-



tanone imine is not detectable. On the other hand, treatment of **33** with sodium hypochlorite produces  $\text{CO}_2$  and the imine. The enzymatic production of a ring expansion product rather than a hydroxylation product or decarboxylation provides strong support for the sequential single-electron-transfer mechanism for ethylene biosynthesis. The reactivity of *N*-heteroatom derivatives of these amino acids is clearly divergent from that of the

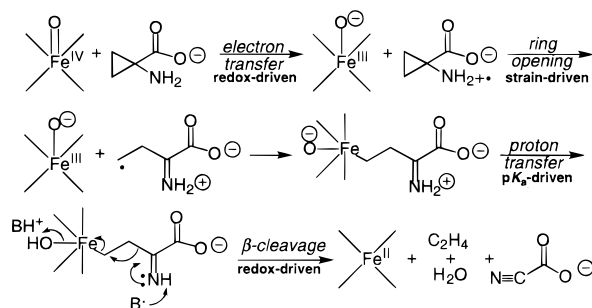


FIGURE 5.

radicals. Since the enzymatic and radical processes give the same product, they must follow the same, i.e., radical, mechanism. The hydroxylation/nitrene/nitrenium mechanism must therefore be discounted.

## Perspective on Non-Heme Iron–Oxo Enzyme Catalysis

A common theme in our analysis of the EFE is that the enzyme functions mainly to provide a potent oxidant to the substrate. We hypothesize that the major influence on the products is the intrinsic chemistry between them, and the role of the enzyme is more passive than usually considered. Admittedly, it provides a constrained and controlled environment in which reactive intermediates such as radicals can be housed, but it is difficult to see what other forces the protein can exert on the postulated reaction pathways. In particular, the Pauling theory<sup>40</sup> conceives of enzymes as having evolved to bind most strongly to the transition state of a catalyzed process. It is clear that this concept cannot apply fully to the non-heme iron enzymes under consideration here, since there are multiple transition states involved in the generation of the oxidizing species, its reaction with substrate, and the subsequent chemistry of intermediates, which may simply reflect the intrinsic reaction pathways of compounds when oxidized. It is difficult to imagine how a small, monomeric non-heme iron enzyme could actively participate in the dozen or so transition states involved in converting oxygen and ACC to ethylene, carbon dioxide, and hydrogen cyanide. The significant successes in the development of bioorganic model systems for both P450-type oxygenases and the EFE support the idea that the chemistry is intrinsic to the substrate–oxidant pair. If EFE is a “non-Pauling” enzyme, a mechanism can be readily suggested for ethylene biosynthesis that exploits purely chemical principles to drive the reaction processes (Figure 5).<sup>41</sup> The role of the protein and its ligands is likely most important in gathering together the substrate and multiple cofactors/reactants, overcoming entropy and generating the oxidizing species. Lessons learned about the activation of oxygen in the EFE thus may be directly applicable to other enzymes in the family. Furthermore, as Benner has pointed out,<sup>42</sup> maximizing an enzyme catalytic rate by binding more tightly the highest transition state is not the only solution to the biological problem of converting substrate to product. Simply making more enzyme may be more expedient. This solution seems to have been

taken in some plants and microorganisms using non-heme iron enzymes. For example, on the basis of the purification factors required to reach homogeneity, clavaminic synthase is a relatively abundant protein in *Streptomyces clavuligerus*,<sup>43</sup> as is the EFE in apples. In concert with the latter datum, Woodson has shown that the EFE mRNA is highly expressed in generative tissue, as measured by a high proportion (up to 25%) of EFE-homologous cDNAs.<sup>44</sup> Because the enzyme is abundant and is committing suicide at a significant rate (vide supra), it is unlikely that inhibiting it would have much effect on plant physiology. The weak antisenescence properties of 1-aminocyclopropanecarboxylic acid (**17**) support this idea.

## Prospects

As investigations concerning the mechanism of the conversion of ACC to ethylene draw to a close, it becomes apparent that we know too little about how the enzyme generates the oxo intermediate presumed to be key to the oxidation chemistry of non-heme iron enzymes. This area is clearly ripe for investigation. Heterologous expression systems,<sup>45</sup> including a collaboration between our laboratory and Dilley's at Michigan State, now permit the isolation of milligram quantities of EFE. Site-directed mutagenesis studies are thereby facilitated that, inter alia, have permitted the unambiguous identification of His<sup>177</sup> and His<sup>234</sup> as the ligands of the essential iron. A breakthrough in crystallographic analysis is also likely soon, with discussion persisting concerning crystals of recombinant EFE. One can also expect future definition of the molecular basis of enzyme inactivation. A remaining unanswered question concerns the mechanism of CO<sub>2</sub> activation. Biophysical studies of the metal site should provide an opportunity for discovery and comparison to the best-known member of the class, IPNS. Finally, elucidation of the determinants of co-oxidant usage (ascorbate,  $\alpha$ -ketoglutarate, or none) in non-heme iron enzymes is a significant challenge that no laboratory has yet addressed.

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